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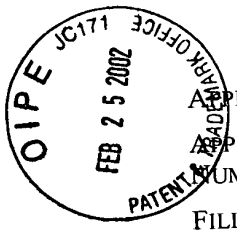

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Boyle *et al.*

APPLICATION NUMBER: 09/672,221 EXAMINER: Jehanne Souaya

FILING DATE: September 27, 2000 ART UNIT: 1655

FOR: *METHODS AND MATERIALS RELATING TO LEUCINE-RICH REPEAT PROTEIN-LIKE (LRR PROTEIN-LIKE) POLYPEPTIDES AND POLYNUCLEOTIDES*

Commissioner for Patents
Washington, D.C. 20231

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RESPONSE TO OFFICE ACTION

This is in response to the Office Action dated November 23, 2001.

Please amend this application as follows:

In the Claims:

Please cancel claims 1-9, 12-24, and 26-30 without prejudice or disclaimer.

Please amend claim 10 as follows:

A1

10. (amended once) An isolated polypeptide comprising the amino acid sequence selected from the group consisting of SEQ ID NOs: 4 and 6-17.

Please add the following claim:

A2

31. (new) An isolated polypeptide comprising an amino acid sequence which is 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 4 and 6-17.

Pursuant to 37 CFR 1.121(c)(1)(ii), a marked up version of the claims showing the changes made appears as Appendix A of this Amendment.

REMARKS

Applicant has amended the claims to clarify and more particularly indicate the claimed subject matter. The amendment is made for the sole purpose of expediting prosecution and not in response to any ground or reason of patentability presented by the USPTO.

Support for the amendment to claim 10, and for new claim 31, appears at page 37, line 1 through line 18. No new matter is added. Accordingly, *claims 10-11, 25 and 31 are in the case.*

Applicants hereby affirm the election without traverse to prosecute the invention of Group III, claims 10-13 and 25. The non-elected claims, having been withdrawn from consideration by the Examiner, have been cancelled herein without prejudice or disclaimer, but Applicants reserve the right to prosecute the same or similar claims in continuing applications.

Applicants also submit herewith an Information Disclosure Statement for consideration by the Examiner. The IDS is accompanied by the fee required by 37 CFR 1.97(c); as such, Applicants respectfully request consideration of the IDS.

Utility

Rejection of claims 10-13 and 25 under 35 U.S.C. §101

Claims 10-13 and 25 were rejected as unpatentable under 35 U.S.C. §101 for lack of a specific or substantial asserted utility, or a well-established utility. The Examiner's position appears to be, in summary, that the invention as claimed lacks a specific or substantial asserted utility because, *inter alia*, the specification allegedly does not teach the biological activity or function of SEQ ID NOS 4 or 17, or where "active domains" are located; the disclosed uses of the claimed polypeptides are not specific; and that the research disclosed in the specification does not constitute a specific and substantial utility. Applicants traverse the Examiner's rejections to the extent they are applied to the claims as amended, and as argued hereinbelow.

Applicants have amended claim 10 to more particularly point out and indicate that claimed invention as a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs 4 and 6-17. Applicants respectfully assert that the polypeptides of the present invention are patentable under 35 U.S.C. §101.

The claimed invention relates to isolated polypeptides comprising the amino acid sequence of, or one 99% identical to, SEQ ID NOs: 4 and 6-17, and compositions and kits containing the polypeptides. The claimed polypeptides show strong homology to members of the LRR protein-like family of proteins, including acid labile subunit (ALS) protein and glycoprotein (GP) Ib and V proteins. These proteins are useful in and of themselves.

For example, the ALS protein is an important component of serum insulin-like growth factor (IGF) regulation *in vivo* (*see, e.g.,* Janosi et al., *J. Biol. Chem.* **274**(33) 23328-23332 (1999), cited in the Information Disclosure Statement accompanying this response). The presence of ALS in the ternary complex containing IGF-I or –II prevents IGF access to target cells. It is also recognized in the art that ALS also is central to preventing the non-specific metabolic effects of the IGFs (such as causing severe hypoglycemia), as IGF bound in the ternary complexes is not able to cross capillary endothelia and activate the insulin receptor. *see, e.g.,* Boisclair et al. *J. Endocrin.* **170** 63-70 (2001), enclosed herewith as indicative of the knowledge in the art. As such, one of ordinary skill in the art reading Applicants' specification would clearly understand from his or her knowledge in the art the utility of proteins

homologous to ALS. For example, therapies utilizing the claimed polypeptides could be developed to correct or ameliorate defects in the processes mediated by, e.g., IGF-I such as growth hormone action (GH); as well as gene therapies to supply the needed polypeptide *in vivo*. Therefore, Applicants' specification clearly demonstrates at least a well-established utility for the claimed invention, meeting the requirements of 35 U.S.C. §101.

The Examiner cited Kobe et al. (*Current Opinion in Structural Biology* **5**:409-416 (1995)) in support of the idea that anything less than 100% sequence identity by the claimed polypeptides to a known polypeptide, e.g., ALS, is insufficient to prove a specific or substantial utility for the claimed polypeptides to one of ordinary skill in the art. It was argued that since Kobe et al. state that known LRR proteins "only" have in common repetitive sequences and involvement in protein-protein interactions, the prediction of putative leucine-rich repeats in the claimed polypeptides does not indicate a specific biological function or activity of the claimed polypeptides, or to specific diseases that can be identified or treated with the polypeptides.

To the contrary, Applicants note that Kobe et al. support the utility of the claimed polypeptides, as part of the LRR protein superfamily. The authors note at p. 412, column 2, that the high conservation of residues at consensus positions throughout the LRR superfamily make it very likely that the structure of LRRs in other proteins (such as the claimed polypeptides) will closely resemble that of the LRRs in ribonuclease inhibitor (RI). RI is, along with other LRR proteins shown in Kobe et al., shown to be involved in protein-protein interactions, *viz*, RI binds/sequesters ribonuclease. The structures of the Kobe-disclosed LRR superfamily proteins also have utility and are submitted to resemble that of the useful RI protein. The claimed polypeptides are members of the LRR superfamily and are strongly similar to known and useful polypeptides, e.g., ALS. Given the teaching of Kobe et al. above, and the similarity of the claimed polypeptides to, e.g., ALS, Applicants respectfully submit that there is sufficient teaching in the to show a utility of the claimed polypeptides to one of ordinary skill in the art.

The Examiner has also taken the position, citing Russell et al. (*J. Mol. Biol.* **244** 332-350 (1994)), that while Applicants' specification teaches that, e.g., SEQ ID NO: 4 has 45% similarity to human glycoprotein V protein, that "It is known for nucleic acids as well at proteins, however, that even a single nucleotide or amino acid change or mutation can destroy the function of the biomolecule in many instances, *albeit not in all cases*. The effects of these changes are largely unpredictable as to which ones have a significant effect versus not." (emphasis added.) To support the position that sequence homology does not necessarily result in the homologous sequences having similar functions, the Examiner cites Russell et al. for the conclusion that "...there is little in common between distantly related protein structures and that secondary structure lengths and loops in distantly related structures vary substantially".

The essence of the Russell paper, however, is *not* that homologous sequences can have different functions, but rather is that proteins which have similar 3D structures *may or may not* have similar primary sequences. Russell et al. analyze the phenomenon that "proteins having no detectable sequence similarity can adopt similar 3D structures." *see* p. 333. Based on their analysis, Russell et al. conclude on page 332 that "proteins having similar 3D structures can have little in common apart from a scaffold of core secondary structures" and that "within proteins having little or no sequence similarity..., structural features frequently had a degree of conservation comparable to dissimilar 3D structures." Russell et al. can not properly be used as a reference to conclude that, e.g., the 45% sequence homology presented by Applicants, means that the claimed invention is not a member of the LRR protein-like family of proteins, since Russell analyzed proteins with 20% homology to study the similarity in 3D structure.

Widely Disparate Amino Acid Sequences Commonly Have Comparable Structures and Biological Functions.

Although the sequences of the present invention *may* be considered to have a low degree of homology with a known protein family (e.g., 44% homology to human ALS protein), the claimed sequences are believed to share comparable three-dimensional structure and biological functions with members of the known LRR protein-like family of proteins such as ALS and human glycoprotein V protein. Based on this shared structure and function, the present sequences can be ascribed a utility similar to that of the LRR protein-like family of proteins. Discussed in detail below are numerous publications that clearly demonstrate that structure and function is preserved among proteins that are members of protein families, even though they may have low sequence similarity.

Strynadka and James (*Annu. Rev. Biochem.* **58**:951-998 (1989)) review the calcium binding domains and the 3D structures of several calcium binding proteins. (Table 1, page 953). The primary structures of several calcium binding proteins in the calcium binding domain are aligned in Table 2 (p. 958) showing the residues providing atoms that bind calcium identified by asterisks using the one-letter amino acid code. Table 2 shows that there is considerable variability in the amino acid residues including those involved in binding calcium as well as in the intervening amino acids. Sequence alignments among the various calcium binding proteins demonstrates that the greatest per cent sequence identity is 51%. Accordingly, proteins with a sequence homology of 51% and lower maintain the calcium binding function of the particular proteins. This review thus demonstrates that in spite of the wide variation in sequence among the proteins (see Table 2) the three dimensional structure and calcium binding function of the various proteins is closely preserved.

Pabo and Sauer (*Annu. Rev. Biochem.* **61**:1053-1095 (1992)) similarly demonstrate that broad variability in the identity of amino acid residues in the sequences of similar domains are found in proteins of similar function. Pabo et al. discuss structural families of transcription

factors and principles of DNA recognition. Several families of domains that bind DNA in response to a stimulus are presented in turn. For example, an alignment among 10 proteins of a helix-turn-helix (HTH) domain is shown in Fig. 1 (p. 1056). This domain is involved in specific DNA recognition and binding, as visualized in many three dimensional structures. The homeodomain is about 60 residues in length and is long enough to form a stable structure on its own (p. 1062). An alignment of nine homeodomains is shown in Fig. 4 (p. 1063), including alpha2 and engrailed. The alpha2 and engrailed homeodomains differ in sequence, but they have a similar structure (p. 1064) and they also dock against DNA in very similar ways (p. 1065).

Pabo et al. also disclose that homologous zinc finger DNA binding domains occur in a broad range of proteins. Fig. 8 shows an alignment of 9 zinc finger domains. The function of binding to a specific DNA site is maintained throughout all the zinc finger domains. Additionally, Pabo et al. show alignments for 9 steroid receptor domains (Fig. 12; p. 1075), 9 leucine zipper DNA binding domains (Fig. 13; p. 1075), helix-loop-helix domains (Fig. 14; p. 1078) and beta sheet DNA binding sequences (Fig. 15; p. 1078). In most of the transcription factor DNA binding domains a small number of certain specific residues are strongly conserved and yet the intervening amino acid residues are highly variant. Up to about 50%, or more, in various alignments, of the residues differ and show little or no degree of identity. Pabo et al. thus demonstrate that broad variability in the identity of amino acid residues in the sequences of similar domains are found in proteins of similar function, and that low amino acid sequence identity still provides similar three dimensional structures and similar DNA binding functions.

Yang and Honig (*J. Mol. Biol.* **301**: 691-711 (2000)) also demonstrate that similar structures can fold without having a set of highly conserved residue clusters or a well-conserved sequence profile. Yang et al. present an objective algorithm for optimally superimposing the three dimensional structures of several proteins. Several figures present sequence alignments of the superimposed proteins as well as the corresponding three dimensional structures superimposed on one another. Aligned amino acid sequences show wide variability in the identity of a residue, including a gap, at a given position, *i.e.*, in general, there is a low extent of identity or similarity. Nevertheless, in the cognate superposition, the spatial alignments of the structures are strikingly similar.

In particular, Fig. 1, panels (a) and (b) show sequence alignments for several immunoglobulin chains, and Fig. 2 shows the superposition of the corresponding three dimensional structures; Figs. 4 and 5 show corresponding comparisons for serine proteinases; Fig. 8 panels (a) and (b) show corresponding comparisons for c-type immunoglobulin-like domains; Fig. 9 panels (a) and (b) show corresponding comparisons for v-type immunoglobulin-like domains; and Fig. 10 panels (a) and (b) show corresponding comparisons

for s/h-type immunoglobulin-like domains. Yang et al. conclude "...the most conserved residues are generally located in the regions where tertiary interactions occur and that are relatively conserved in structure. Nevertheless, the conservation patterns are relatively weak in all cases studied, indicating that structure-determining factors that do not require a particular sequential arrangement of amino acids, such as secondary structure propensities and hydrophobic interactions, are important in encoding protein fold information. In general, we find that similar structures can fold without having a set of highly conserved residue clusters or a well-conserved sequence profile..." (page 691, Abstract; emphasis added).

Thus the analysis of Yang and Honig provides extensive support for the position adopted by Applicant that extent of sequence identity between the claimed sequences and the known protein sequences identified in BLAST searches (see FIGs 1-4 of Applicants' specification) is sufficient to preserve three dimensional folding among members so identified, and thereby to maintain essential functional attributes common to the proteins in question.

Applicants submit that the evidence presented herein demonstrates that wide variation in amino acid sequence among members of families can still provide similar three dimensional structures and preservation of similar function. The extent of identity between the claimed proteins and the related proteins that are members of the known LRR protein-like family of proteins is well within the extent of identity in the examples discussed by Strynadka, Pabo, and Yang in which structure and function are retained. Reconsideration and withdrawal of the rejection under 35 U.S.C. §101 is therefore respectfully requested.

Enablement

Rejections of claims 10-13 and 25 under 35 U.S.C. §112, first paragraph

The Examiner rejected Claims 10-13 and 25 under 35 U.S.C. § 112, first paragraph, because one of ordinary skill in the art would allegedly not know how to use the invention since the claimed invention was said to not be supported by either a specific or substantial asserted utility, or a well-established utility as argued by the Examiner in the 35 U.S.C. §101 rejection. Applicants traverse to the extent the rejection applies to the claims as amended.

Applicants have disclosed the sequences for the claimed subject matter. One of skill in the art would know how to make the claimed invention based on the disclosed sequence. As described above, the sequences of the pending claims have a specific, substantial, and credible utility. Since the subject matter of the pending claims has been demonstrated as above as having utility, Applicants respectfully submit that the claims are properly enabled, and as such, the rejection is moot. Applicants therefore respectfully request that the rejection under 35 U.S.C. §112, first paragraph be withdrawn.

The Examiner also rejected Claims 10-13 and 25 under 35 U.S.C. § 112, first paragraph, because the specification was said to not reasonably provide enablement for isolated

polypeptides that are at least 80% identical to the claimed amino acid sequences. Applicants traverse to the extent the rejection applies to the claims as amended. The claims have been amended herein to clarify that the claimed polypeptides comprise the amino acid sequence of, or one 99% identical to, SEQ ID NOs: 4 and 6-17, as well as compositions and kits containing the polypeptides. These claims are fully supported by the specification, e.g., Example 3, detailing the elucidation and/or preparation of claimed polypeptides including SEQ ID NO: 4, and Example 4, showing how SEQ ID NO: 4 is expressed in CHO cells. In view of the above, Applicants respectfully submit that the specification fully supports the claimed invention, as amended herein, and therefore respectfully request the rejection under 35 U.S.C. §112, first paragraph be reconsidered and withdrawn.

Written Description

Rejections of claims 10-13 and 25 under 35 U.S.C. §112, first paragraph

The Examiner rejected Claims 10-13 and 25 under 35 U.S.C. §112, first paragraph, as containing subject matter not described in the specification in a way to reasonably convey to one skilled in the art that Applicants had possession of the claimed invention at the time the application was filed. In the rejection, the Examiner noted that (p)olypeptides comprising SEQ ID NOS. 4 and 17, and SEQ ID NOS. 6-16, met the written description requirements of 35 U.S.C. §112, first paragraph. The presently amended claims are directed to this subject matter. New claim 31 is respectfully submitted to meet muster under the written description requirement as well. As such, Applicants respectfully submit that the presently amended claims moot the rejection under 35 U.S.C. §112, first paragraph, and withdrawal of the rejection is believed to be in order and requested.

Utility

Rejections of claims 10-13 and 25 under 35 U.S.C. §102(b)

Claims 10-13 and 25 were rejected under 35 U.S.C. §102(b) as anticipated by Hardiman et al. WO 98/50545 ("Hardiman"). Applicants traverse to the extent it applies to the claims as amended herein. The presently amended claims are not anticipated by Hardiman, i.e., the cited Hardiman SEQ ID NO: 12. Since the present amendment has made the rejection over Hardiman moot, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. §102(b).

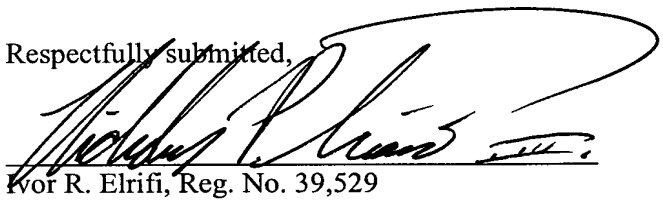
Claim 13 was rejected under 35 U.S.C. §102(b) as anticipated by Miller et al. U.S. Patent No. 5,298,239 ("Miller"). As claim 13 has been cancelled herein, the rejection is moot. Applicants respectfully request withdrawal of the rejection.

SUMMARY

On the basis of the foregoing amendments, Applicants respectfully submit that the pending claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact either of the undersigned at the telephone number provided below.

Please charge any underpayments or credit any overpayments associated with this communication to our Deposit Account No. 50-0311, reference 24059-026. A duplicate of this letter is enclosed.

Respectfully submitted,



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Dated: February 25, 2002

REVIEW

The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system

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Abstract

The insulin-like growth factors-I and -II (IGFs) are involved in a wide array of cellular processes such as proliferation, prevention of apoptosis, and differentiation. Most of these effects are mediated by the IGF-I receptor, although at higher IGF concentrations the insulin receptor can also be activated. As the expression of both the IGFs and their receptors is widespread, IGFs are thought to have autocrine/paracrine modes of actions also, particularly during foetal life. The endocrine component of the IGF system is recognised to be important after birth, with IGF-I mediating many of the effects of growth hormone (GH), and linking anabolic processes to nutrient availability. Consideration of ligands and receptors, however, is insufficient to provide a complete understanding of the biology of IGF. This is because IGFs are found in binary complexes of 40–50 kDa with members of a family of IGF-binding proteins (IGFBPs-1 to -6) in all biological fluids. In addition, in postnatal serum, most IGFs are sequestered into ternary complexes of 150 kDa consisting of one molecule each of IGF, IGFBP-3 or IGFBP-5, and acid-labile subunit (ALS). Despite evidence that ALS plays an important role in the biology of circulating IGFs, it has received only limited attention relative to the other components of the IGF system. This review provides an overview on the current knowledge of ALS protein and gene structure, organisation and regulation by hormones, and insights from novel animal models such as the ALS knockout mice.

Journal of Endocrinology (2001) **170**, 63–70

Introduction

Insulin-like growth factors (IGFs)-I and -II play crucial roles in growth and development. IGF-I is synthesised by a variety of cell types, and is involved in linear growth, cell proliferation and differentiation, and in apoptosis. Most of these effects are mediated by both endocrine and autocrine/paracrine mechanisms through the IGF-I receptor (Werner *et al.* 1991). In circulation, almost all the IGFs are present as 150 kDa ternary complexes comprising of one molecule each of IGF, IGF-binding protein-(IGFBP)-3 (the predominant IGFBP in serum) or IGFBP-5, and a 85 kDa glycoprotein, the acid-labile subunit (ALS) (Rechler 1993, Baxter 1994, Ooi & Boisclair 1999). Plasma also contains lower molecular mass complexes of ~50 kDa which are made up of several IGFBP species (IGFBPs-1, -2, -4, and -6) that are incompletely saturated with IGFs leaving virtually no free IGFs in circulation (Jones & Clemmons 1995, Stewart & Rotwein 1996). Unlike free IGFs and IGFs bound to the ~50 kDa binary complexes, which can cross the vascular endothelium, formation of the ternary complexes restricts the IGFs to the circulation, prolongs their half-lives and allows them to be stored at high concentration in plasma to facilitate their endocrine actions and to minimise their local effects due to their intrinsic insulin-like activities such as hypoglycaemia (Zapf *et al.* 1995). Despite this important role of ALS in determining the endocrine effects of IGFs on target tissues, it has historically received limited attention compared with the other members of the circulating IGF system, such as the IGFBPs and proteases. ALS, synthesised exclusively by the liver, is predominantly stimulated by growth hormone (GH), as are both IGF-I and IGFBP-3. Presence of ALS after birth is coincident

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with increased responsiveness to GH resulting from an increase in GH secretion and hepatic GH receptors, and is an important factor driving the formation of the 150 kDa complex. After the initial increase in ALS after puberty, ALS concentrations largely remains unchanged throughout adulthood, thereby ensuring that IGFs are constantly sequestered as the 150 kDa complexes. With the recent characterisation of the ALS gene in many species, including structure–function relationships, and elucidation of the regulation of ALS synthesis, future research efforts can now be devoted to understanding the significance and health benefits of the recruitment of IGFs into ternary complexes during postnatal life. This review summarises recent work by others and us on the biochemical properties of ALS, its gene structure and organisation, the regulation of its synthesis by GH and the molecular mechanisms underlying this transcriptional regulation. New information obtained from our studies on the ALS knockout mice will also be discussed.

ALS gene and protein structure

The *ALS* gene was first cloned in 1996, in the mouse (Boisclair *et al.* 1996). The mouse gene covers approximately 3·3 kilobases (kb) of chromosomal DNA, and is comprised of two exons separated by a 1126 base pair (bp) intron. Exon 1 encodes the first five amino acids of the signal peptide, and exon 2 encodes the remaining 22 amino acids of the signal peptide and the 576 amino acid residues of the mature protein. This chromosomal structure is conserved across species, as shown by the subsequent descriptions of the gene in rat, man and sheep (Delhanty & Baxter 1997, Rhoads *et al.* 2000, Suwanichkul *et al.* 2000). *ALS* is a single-copy gene, and was mapped to bands A2-A3 of mouse chromosome 17, and to the short arm of human chromosome 16 at p13·3 (Boisclair *et al.* 1996, Suwanichkul *et al.* 2000).

In all species studied so far, transcription of the *ALS* gene is controlled by a TATA-less promoter and produces mRNAs of ~2·2 kb (Boisclair *et al.* 1996, Rhoads *et al.* 2000, Dai & Baxter 1992, Leong *et al.* 1992, Delhanty & Baxter 1996). These mRNAs encode proteins ranging in size from 603 amino acids in the mouse to 611 residues in the sheep. Identity of mature ALS is 93% between mouse and rat, 79% between mouse and human, and 73% between mouse and sheep. Structural features almost completely conserved across species include the presence of 12–13 cysteine residues, six or seven asparagine-linked glycosylation sites, and 18–20 repeating leucine-rich domains of 24 amino acids. These leucine-rich domains account for approximately 75% of the mature protein, and organise ALS into a doughnut-shaped structure (Janosi *et al.* 1999*b*). ALS belongs to the superfamily of leucine-rich repeats – a family characterised by their ability to participate in protein–protein interactions.

Biochemical attributes of ALS

Human ALS has an apparent molecular weight of 84–86 kDa after purification and of 66 kDa after enzymatic deglycosylation (Baxter *et al.* 1989, Baxter & Dai 1994). ALS has no affinity for free IGF-I or IGF-II and very low affinity for uncomplexed IGFBP-3. It, however, readily binds to binary complexes of IGF and IGFBP-3 (Baxter & Martin 1989, Twigg & Baxter 1998). Affinities of ALS for binary complexes are 300–1000-fold lower than those of IGFBP-3 for IGF-I or -II (Holman & Baxter 1996). As suggested by its name, the ability of ALS to form ternary complexes is irreversibly destroyed under acidic conditions (pH<4·5; Holman & Baxter 1996).

IGFBPs-1, -2, -4 or -6 cannot substitute for IGFBP-3 in forming the ternary complex with ALS. In contrast, IGFBP-5, the member of the IGFBP family most closely related to IGFBP-3, is able to form ternary complexes with ALS, and circulates predominantly in a high molecular weight form characteristic of a ternary complex with IGF-I or -II and ALS (Twigg & Baxter 1998). Ternary complexes containing IGFBP-5 account at best for ~10% of total ternary complexes in serum, and their significance remains unclear. In addition, unlike IGFBP-3, IGFBP-5 is able to associate weakly with ALS in the absence of IGFs, raising the possibility that a large fraction of the high molecular weight complexes containing IGFBP-5 do not carry any IGF (Twigg *et al.* 1998).

Structurally, IGFBPs-1 to -6 share homologous amino- and carboxy-terminal domains but have unique central domains (Rechler 1993, Ooi & Boisclair 1999). Domain swapping experiments with IGFBP that are unable to form ternary complexes (i.e. IGFBPs-2 and -6) have demonstrated that the carboxy-terminal domains of IGFBPs-3 and -5 are important for binding ALS (Hashimoto *et al.* 1997, Twigg *et al.* 1998). Binding ability was further mapped to a conserved region of 18 amino acid residues corresponding to residues 201–218 in IGFBP-3, and residues 215–232 in IGFBP-5 (Firth *et al.* 1998, Twigg *et al.* 1998). This region is composed of mostly basic and positively charged amino acid residues. More recently, the central domain of IGFBP-5 was also shown to bind ALS, even in the absence of the carboxy-terminal domain (Twigg *et al.* 2000).

In the case of ALS, recent studies have sought to determine the role played by the sugar residues. Removal of the negatively charged sialic acid from the glycan chains of ALS reduces the affinity of ALS for the IGF-I and -II binary complexes, but does not eliminate complex formation (Janosi *et al.* 1999*a*). Independent mutations of each of the seven *N*-linked glycan attachment sites of human ALS do not eliminate its ability to form ternary complexes with IGFBP-3, but complete deglycosylation does. Overall, these data are consistent with a model in which the positively charged, conserved domain of 18 amino acid present in IGFBP-3 and -5 interacts with negatively

charged regions of ALS. This is supported by molecular modelling of ALS, which predicts two densely negatively charged regions, the first one created by the clustering of six of the seven *N*-linked sugar chains, and the second by the amino acids present at the internal surface of the doughnut-shaped protein (Janosi *et al.* 1999*b*).

Regulation of ALS synthesis

ALS is found in high concentration almost exclusively in postnatal serum (Baxter 1990*a*, Khosravi *et al.* 1997). Typical concentrations in human and rat serum are 230 and 570 nM respectively. ALS circulates in excess over the other components of the ternary complex, with 50–60% of serum ALS found in free form (Baxter 1990*a*, Baxter & Dai 1994, Khosravi *et al.* 1997). When total RNA is analysed by northern blot in rat, primate and sheep, *ALS* gene expression can be detected only in liver (Dai & Baxter 1994, Delhanty & Baxter 1996, Rhoads *et al.* 2000). Synthesis in liver is confined to parenchymal cells (Chin *et al.* 1994). Immunoreactive ALS is also present at very low concentration in cerebrospinal fluid, amniotic fluid, milk and lymph, and at low to medium concentrations in peritoneal, synovial, ovarian and blister fluid (Baxter 1990*a*, Xu *et al.* 1995, Cwyfan Hughes *et al.* 1997, Khosravi *et al.* 1997, Labarta *et al.* 1997). Serum is probably the source of most of this extravascular ALS, although local synthesis may occur in some tissues. Using sensitive methodologies (e.g. *in situ* hybridisation, generation of expressed sequence tags), *ALS* gene expression is detected in some extrahepatic tissues, including kidney, developing bone, lactating mammary gland, thymus and lung (Chin *et al.* 1994, Janosi *et al.* 1999*c*). ALS mRNA has also been detected in the theca and granulosa cells of the porcine ovary (Wandji *et al.* 2000). Extravascular ALS may be particularly significant in the ovary, because IGFs are found almost exclusively in 150 kDa complexes in human follicular fluid (Cwyfan Hughes *et al.* 1997). Irrespective of its origin, extravascular ALS can modulate local IGF action through sequestration of IGFs into ternary complexes, as recently shown by the ability of ALS to potentiate the inhibitory effects of IGFBP-5 on thyroidal cell proliferation (Twigg *et al.* 1999). This inhibitory role of ALS on IGF actions is consistent with the observation that generalised overexpression of ALS caused 13% growth retardation in 4- and 8-week-old mice (Modric *et al.* 1999).

Onset of ALS synthesis is one of the last events in the development of the circulating IGF system. In humans, ALS is undetectable in foetal serum at 27 weeks of gestation, but is present at term (Lewitt *et al.* 1995). Serum concentrations of ALS increase fivefold from birth to puberty, and decline somewhat in older individuals (Baxter 1990*a*). Studies in rats have shown that an induction of *ALS* gene expression in liver is responsible for this increase in plasma ALS in early life (Baxter & Dai 1994, Dai & Baxter 1994, Frystyk *et al.* 1998). In the sheep, abundance of ALS mRNA is also low before birth, but increases abruptly within 7 days of postnatal life (Rhoads *et al.* 2000). The functional consequence of this pattern of ALS expression in the sheep is that IGFs circulate primarily in 50 kDa complexes before birth, and in 150 kDa complexes 1 week after birth (Butler & Gluckman 1986).

GH is by far the most potent inducer of ALS mRNA in liver and of ALS in plasma (Baxter 1990*a*, Baxter & Dai 1994, Ooi *et al.* 1997, Olivecrona *et al.* 1999). The importance of this regulation is underlined by the near complete absence of ALS in GH-deficient states (Zapf *et al.* 1989, Gargosky *et al.* 1994, Aguiar-Oliveira *et al.* 1999), and by the temporal correlation between appearance of ALS mRNA and functional GH receptor in liver of both sheep and rats (Gluckman *et al.* 1983, Tiong & Herington 1992). These effects of GH in liver are direct and occur at the level of *ALS* gene transcription (Ooi *et al.* 1997, 1998).

A variety of conditions has been shown to reduce serum ALS in rats and humans. They include fasting, undernutrition and catabolic diseases such as diabetes, burn injury and cirrhosis (Dai & Baxter 1994, Bereket *et al.* 1996, Lang *et al.* 1996, 2000, Oster *et al.* 1996, Fukuda *et al.* 1999, Moller *et al.* 2000). Negative regulation of ALS synthesis occurs at both transcriptional and post-transcriptional levels. Dexamethasone, cAMP and epidermal growth factor decrease secretion of ALS in primary rat hepatocytes, primarily by reducing the abundance of ALS mRNA (Dai *et al.* 1994, Delhanty & Baxter 1998). Increases in factors such as glucocorticoid and cellular cAMP could explain the marked decrease in ALS synthesis observed during thermal injury and liver failure (Lang *et al.* 2000, Moller *et al.* 2000). In contrast, insulin deficiency may be the primary defect causing decreased concentrations of serum ALS during fasting, undernutrition and diabetes. This effect of insulin occurs post-translationally, because insulin increases ALS secretion in the absence of any change in ALS mRNA abundance in primary hepatocytes (Dai *et al.* 1994). Finally, decreased ALS synthesis could also occur secondarily to the development of GH resistance in liver. Recent studies have shown that most of the negative actions of cAMP and the inflammatory cytokine interleukin (IL)-1β on ALS synthesis occur via the induction of a GH-resistant state in liver cells (Delhanty 1998, Boisclair *et al.* 2000).

Transcriptional regulation of *ALS* gene in liver

In vivo, GH is the predominant determinant of ALS synthesis, underlying the importance of the IGF system in mediating the growth-promoting effects of GH (Daughaday & Rotwein 1989, Etherton & Bauman 1998). Using rat liver cells, a GH-responsive promoter was

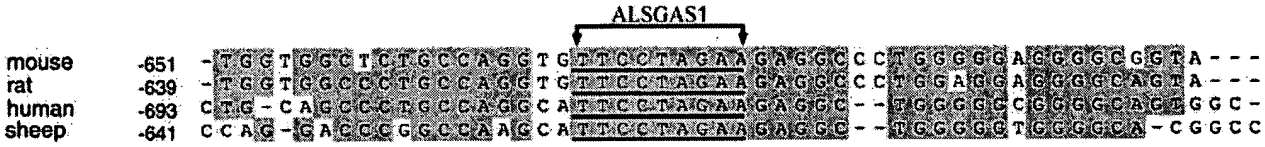

Figure 1 The GH response element of the *ALS* gene is conserved in various species. A 50 bp nucleotide sequence is presented for the mouse, rat, sheep and human ALS promoter (Boisclair *et al.* 1996, Delhanty & Baxter 1997, Rhoads *et al.* 2000, Suwanichkul *et al.* 2000). The position of the most 5′ nucleotide (relative to ATG, +1) is given on the left of each sequence. Areas of identity between sequences are shaded. ALSGAS1, the GH response element (TTCCTAGAA) of the mouse ALS promoter, is underlined. This sequence is conserved in position and in sequence across species.

identified in the genomic fragment corresponding to nt − 2001 to nt − 49 (relative to $A_{+}1TG$) of the mouse *ALS* gene (Ooi *et al.* 1997). The GH-response element of the mouse promoter was located by deletion and mutation analysis to a 9 bp sequence located between nt −633 and −625 (Ooi *et al.* 1998). This sequence was called ALSGAS1 because of its resemblance with the consensus sequence for γ-interferon activated sequence (GAS) (Schindler & Darnell 1995). The effects of GH on the *ALS* gene are mediated by the JAK-STAT pathway: the tyrosine kinase JAK2 is recruited to the activated GH receptor complex and phosphorylates signal transducers and activators of transcription (STAT)-5a and STAT-5b (Schindler & Darnell 1995, Carter-Su *et al.* 1996). After dimerisation, STAT5 isomers translocate to the nucleus, and activate *ALS* gene transcription by binding to the ALSGAS1 element (Ooi *et al.* 1998). The GH signalling pathway leading to increased *ALS* gene transcription is critically dependent on the activation of STAT5 isomers, and is independent of RAS activation, as cells co-transfected with either dominant negative STAT-5a or STAT-5b completely abolished GH stimulation, whereas co-transfection with either dominant negative RAS or constitutively active RAS had no effect (Boisclair *et al.* 1998).

Validation of this mechanism of GH activation of the *ALS* gene was obtained from studies of the human and sheep *ALS* genes. Despite limited homology between their proximal sequence, the mouse, rat, sheep and human genes share complete conservation in sequence and position of the ALSGAS1 element (Fig. 1) (Rhoads *et al.* 2000, Suwanichkul *et al.* 2000). Sheep and human ALS promoters are also GH-responsive when transfected in liver cells, and this responsiveness also requires the presence of the ALSGAS1 element (Rhoads *et al.* 2000, Suwanichkul *et al.* 2000). Overall, these observations suggest conservation of the mechanism by which GH stimulates transcription of the *ALS* gene.

IL-1β blocks the GH-dependent induction of ALS and IGF-I mRNA in primary hepatocytes (Wolf *et al.* 1996, Thissen & Verniers 1997, Delhanty 1998, Barreca *et al.* 1998, Boisclair *et al.* 2000). Some of these effects have been attributed to the down-regulation of the GH receptor by IL-1β (Wolf *et al.* 1996, Thissen & Verniers 1997). Using ALS as a model of GH-regulated gene transcription, IL-1β was also shown to interfere with the activation of STAT5 (Boisclair *et al.* 2000). This interference is mediated by the intracellular suppressor of cytokine signalling (SOCS)-3, an inhibitor of the JAK-STAT pathway. This may be an important mechanism that contributes to an apparent GH-resistance seen in inflammatory diseases.

Physiological role of ALS in the circulating IGF system

In adult animals, serum IGFs reach concentrations that are ~1000 fold that of insulin. ALS is a critical component that contributes to the development of this large reservoir by extending the half-lives of IGFs from 10 min when in free form, and 30–90 min when in binary complexes, to more than 12 h when bound in ternary complexes (Guler *et al.* 1989, Zapf *et al.* 1995). Given this large reservoir of bioactive IGFs, a second important role of ALS is the prevention of the non-specific metabolic effects of the IGFs, such as causing severe hypoglycaemia (Zapf *et al.* 1995). ALS is able to contain these metabolic effects because IGFs in ternary complexes cannot traverse capillary endothelia and activate the insulin receptor, whereas free IGFs and IGFs bound as binary complexes can do so. Incorporation of IGFs into ternary complexes therefore completely restrains the intrinsic insulin-like effects of the IGFs (Zapf *et al.* 1995). An important correlate of this model is that specific mechanisms must exist to release IGFs from ternary complexes for their actions on target cells. Proteolytic attack of IGFBP-3 and interactions of the ternary complex with proteoglycans have been shown to release IGFs (Baxter 1990*b*, Lee & Rechler 1996). It is also possible that much of the released IGFs is the product of the equilibration between the ternary complex and its individual components in serum.

These roles of ALS have been inferred from short-term studies of GH-deficient animals (Zapf *et al.* 1989, Gargosky *et al.* 1994). However, in these animals, the concentration of all the components of the ternary complex are simultaneously decreased, making it difficult to delineate the separate roles of ALS from those of IGF-I and

IGFBP-3. Moreover, studies covering the entire life of GH-deficient animals are usually not feasible. For these reasons, we have generated an ALS-null mouse model in which the ternary complexes are absent due to the inactivation of the *ALS* gene (Ueki *et al.* 2000). Interestingly, null ALS mice have dramatically reduced circulating IGF-I and IGFBP-3 concentrations compared with their wild-type siblings (62 and 88% reductions respectively). These changes occur despite the absence of any reductions in IGF-I or IGFBP-3 synthesis, as expression of both these genes in liver, the predominant site of synthesis, was normal. These results proved that ALS is absolutely necessary for the serum accumulation of both IGF-I and IGFBP-3 and that, without ALS, induction of IGF-I and IGFBP-3 synthesis after birth would cause only a modest increase in their plasma concentrations (Albiston & Herington 1992, Kikuchi *et al.* 1992).

Under normal circumstances, ALS is usually not considered to play a role in regulating serum IGFs, because it circulates in large excess over the concentrations of IGFs and IGFBP-3. This notion needs to be reconsidered in view of the low association constant of ALS for the binary complexes of IGFBP-3 and IGF (Holman & Baxter 1996). Mice with a single null ALS allele provide an example of this phenomenon. They secrete less ALS, which results in significant reductions in serum IGF-I (17%) and IGFBP-3 (40%) (Ueki *et al.* 2000). Another example is provided by GH treatment of normal animals. A greater concentration of serum IGF-I probably represents the combined effect of increased hepatic synthesis of IGF-I and ALS, whereas greater concentration of IGFBPs-3 and -5 must primarily reflect stabilisation by ALS (Cohick *et al.* 1992, Powell *et al.* 1999).

Despite these disturbances in the circulating IGF system, null ALS animals suffered only a 13% growth deficit by adulthood. This modest effect is surprising, given the central role postulated for plasma IGF-I in regulating postnatal growth (Baker *et al.* 1993, Louvi *et al.* 1997, Etherton & Bauman 1998). However, it is consistent with the observation that abrogation of IGF-I synthesis only in liver, which results in a reduction in plasma IGF-I similar to that of the null ALS mice, does not alter postnatal growth (Sjogren *et al.* 1999, Yakar *et al.* 1999). A revised somatomedin hypothesis that accommodates these findings is one in which the primary function of liver is to supply the IGF-I needed to respond to various challenges. In this model, ALS plays a critical role by capturing liver-derived IGF-I into long-lived ternary complexes. This model would predict that null ALS mice with limited retention of liver derived IGF-I would not respond as well to GH therapy or would have greater muscle wasting after exposure to endotoxin (Clark *et al.* 1995, Frost & Lang 1999). In support of this hypothesis, IGF-I therapies are more effective when ternary complexes formation is enhanced by co-administration of GH or IGFBP-3 (Kupfer *et al.* 1993, Bagi *et al.* 1995).

The null ALS mice have normal concentrations of plasma glucose and insulin, although ternary complexes cannot form (Ueki *et al.* 2000). This is surprising, given the hypoglycaemic potential of IGFs. One reason why hypoglycaemia does not develop in ALS-null mice may be the near absence in mice of serum IGF-II (Wolf *et al.* 1994), a much more potent insulin receptor agonist than IGF-I (Frasca *et al.* 1999). In mammals with high concentration of serum IGF-II, such as humans, the presence of ALS and formation of ternary complexes is likely to play a more important role in containing the intrinsic and more potent insulin-like effects of IGF-II. Indeed, hypoglycaemia in humans suffering from non-islet tumour hypoglycaemia is associated with high concentration of incompletely processed IGF-II and depressed ternary complex formation (Daughaday & Kapadia 1989, Baxter *et al.* 1995). Finally, protection afforded by ALS may extend to the mitogenic effects of IGFs also. Evidence consistent with this hypothesis includes increased incidence of tumours in transgenic mice overexpressing IGF-II (Rogler *et al.* 1994), defects promoting greater concentrations of IGF-II in some cancer cells (Toretsky & Helman 1996, De Souza *et al.* 1997), and the positive association between concentrations of plasma IGF-I and incidence of prostate and breast cancers in human populations (Rosen & Pollak 1999). Although these studies do not establish a cause-and-effect relationship, it is possible that the larger pool of bioavailable IGFs in ALS-null mice would promote excessive cell proliferation and, perhaps, the development of cancers.

Conclusion

Progress on the biology of ALS has been rapid in the past few years. Significant advances include characterisation of the *ALS* gene in many species, resolution of important structure–function relationships and identification of primary mechanisms regulating ALS synthesis. The challenge is now to understand the functional significance of ternary complexes and, more broadly, the role of plasma IGFs after birth. Future efforts need to take into account recent experiments in the mouse showing that liver-derived IGF-I is dispensable for postnatal growth under idealised conditions (Sjogren *et al.* 1999, Yakar *et al.* 1999). These results suggest that the benefits of a large and stable reservoir of plasma IGFs are likely to be more subtle than originally thought, and to vary according to species, development and disease.

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